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Tissue-Specific Transgenic Knockdown of Fos-Related Antigen 2 (Fra-2) Expression Mediated by Dominant Negative Fra-2

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Fos-related antigen 2 (Fra-2) is a member of the Fos family of immediate-early genes, most of which are rapidly induced by second messengers. All members of this family act by binding to AP-1 sites as heterodimeric complexes with other proteins. However, each appears to have a distinct role. The role and biology of Fra-2 are less well understood than those of its relatives c-Fos, Fra-1, and FosB; moreover, Fra-2 target genes remain largely unknown, as does the basis of its selective effects on transcriptional activity. To pursue these issues, we created a transgenic rat line (NATDNF2) in which a dominant negative *fra-2* (DNF2) gene is strongly expressed in the pineal gland; tissue selectivity was achieved by putting the DNF2 gene under the control of the rat arylalkylamine *N*-acetyltransferase (AANAT) regulatory region, which targets gene expression to a very restricted set of tissues (pineal gland ≫ retina). Expression of AANAT is normally turned on after the onset of darkness in the rat; as a result, pineal DNF2 expression occurs only at night. This was associated with marked suppression of the nocturnal increase in *fra-2* mRNA and protein levels, indicating that DNF2 expression inhibits downstream effects of Fra-2, including the maintenance of high levels of *fra-2* gene expression. Analysis of 1,190 genes in the NATDNF2 pineal gland, including the AANAT gene, identified two whose expression is strongly linked to *fra-2* expression: the genes encoding type II iodothyronine deiodinase and nectadrin (CD24).

Fos-related antigen 2 (Fra-2) is a member of the Fos family of transcription factors (9, 24, 34). Members of this family act by forming heterodimeric complexes with Jun proteins, which control gene expression through interaction with the activator protein 1 (AP-1) DNA consensus element (36, 46). In addition, Fos family members can also form heterodimers with other partners, such as some ATF/CREB family members, thereby increasing the number of potential Fra target genes (15). Although significant advances have been made toward understanding the general mechanisms through which Fos family members act (12), little is known about what links any one member of this family with a specific target gene. This is especially true of Fra-2, whose function and biology remain poorly understood. A role in organogenesis is suggested by the robust and distinct pattern of Fra-2 expression that occurs during early development (6, 25); such a role might explain the absence of *fra-2* knockouts, which might be developmentally lethal. A role in short-term regulation of gene expression is suggested by waves of Fra-2 expression in specific adult cells (22, 34, 45).

The short-term role that Fra-2 plays in stimulus-driven gene expression has received significant attention. From this, an outline of the common features of Fra-2 expression has emerged. First, *fra-2* expression is turned on by second messengers, including cyclic AMP (cAMP) (1, 37) and Ca^{2+} (24). Second, the ensuing response is rather protracted (1, 45), albeit less so than the one displayed by the *fra-1* gene (50). Third, Fra-2 protein is modified extensively, primarily through extracellular signal-regulated kinase/mitogen-activated protein kinase (MAPK) phosphorylation (7, 11, 28, 30). Fourth, Fra-2 can activate transcription; however, the strength of this effect appears to be determined by the heterodimerization partner (25, 38) and/or the extent of its phosphorylation (28).

Whereas some general features of Fra-2 expression are now becoming evident, little is known about the basis of Fra-2 selectivity and which genes it regulates. This reflects the absence of in vivo models required to study Fra-2 in a physiologically relevant environment. Here we have evaluated an in vivo approach, in which a dominant negative (DN) version of *fra-2* (DNF2 gene) is expressed in a tissue-specific manner, with the intention of avoiding the deleterious effects likely to result from the global suppression of Fra-2 expression. The pineal gland was selected as a target because methods exist to generate transgenic rat strains in which genetic material is expressed primarily in this tissue (4) and because the *fra-2* gene is physiologically expressed at night in this tissue in a dramatic ~200-fold wave, whereas the levels of other Fos family members remain relatively constant (1). In addition, the rat pineal gland is attractive because it is composed of a nearly homogeneous population of cells, pinealocytes, which simplifies analysis and interpretation.

The nocturnal pattern of Fra-2 expression appears to be unique in the pineal gland because other members of the Fos family fail to respond to the onset of darkness, providing reason to suspect that Fra-2 might function to control rhythmic expression of one or more genes relevant to pineal function. The 24-h pattern in pineal activity is driven by the biological clock in the suprachiasmatic nucleus, which is linked to the

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pineal gland by a multisynaptic pathway; neural regulation of pinealocytes is mediated by the release of norepinephrine and the resulting increase in cAMP and Ca^{2+} (21). This system controls rhythmic expression of genes encoding Fra-2, the melatonin rhythm enzyme–arylalkylamine *N*-acetyltransferase (AANAT) gene, and other rhythmically expressed proteins, all of which are candidates for regulation by a Fra-2-containing complex.

As described here, we generated transgenic rat lines in which a DN version of Fra-2 (DNF2 protein) is expressed in the pineal gland; tissue selectivity was conferred by using the AANAT promoter (4). Expression of the DNF2 gene had a marked suppressive effect on Fra-2 expression, thereby providing a model in which effects of Fra-2 are inhibited in two ways—one via suppression of endogenous Fra-2 levels and the second via competitive effects of the DNF2 protein on the actions of heterodimeric complexes. Analysis of gene expression revealed that Fra-2 appears to play divergent roles in regulating expression of specific genes in the pineal gland. The results are of broad interest because they demonstrate the utility of expressing the DNF2 gene under the control of a tissue-specific promoter to determine the role Fra-2 plays in stimulus-driven gene expression.

MATERIALS AND METHODS

Production of transgenic rats. The NATDNF2 vector was generated using the partial sequence corresponding to the rat Fra-2 (rFra-2) coding sequence between nucleotides 1 and 621 (first 207 amino acids [aa]; GenBank accession no. U18913), which was inserted immediately downstream of the rat AANAT promoter region (Fig. 1A and B, diagrams) (2). Transgenic rats were generated (31) by microinjecting rat embryos with a purified restriction fragment carrying the AANAT promoter and the DNF2 sequence, which had been excised from the NATDNF2 vector by digestion with *Xho*I and *Hpa*I. Transgenic offspring were identified by Southern blot probing (31) of genomic DNA cut with *Pst*I; the probe was an *Xho*I/*Bam*HI fragment of the transgene. This permits detection of an internal 1.7-kb fragment of the incorporated transgene and an ~2.5-kb band representing the endogenous AANAT gene. Note that the latter band alone is detected in the wild type (WT). Copy number determinations were obtained by densitometric analysis (ImageMaster 3.0; Amersham-Pharmacia, Piscataway, N.J.) of transgene-specific bands relative to the level of endogenous gene bands in equivalent lanes. Transgenic mice were maintained as hemizygous lines, and nontransgenic littermates were used as controls in phenotypic analysis.

Transient transfection assay. NIH 3T3 cells were transfected in 24-well plates (200 μl) 42 h prior to harvest. The transfection cocktail contained 5 μl of Lipofectamine and 10 μl of Plus reagent (Life Technologies, Rockville, Md.), 20 ng of a luciferase reporter construct that is driven by a 1.3-kb *Hin*dIII-*Bam*HI upstream regulatory fragment derived from the rat *fra-2* promoter and that contains two AP-1 sites (kindly provided by Anders Molven, Haukeland University Hospital, Bergen, Norway), and a mammalian vector (pCDNA3.1) driving expression of full-length or DN Fra-2. Duplicate transfected cultures were stimulated by addition of 1 mM dibutyryl cAMP (DB_2cAMP) 24 h later. Luciferase activity was measured 18 h later by standard procedures (luciferase assay system; Promega, Madison, Wis.). Results of transient transfection assays are representative of three independent experiments. Statistical analysis was performed by a Student *t* test for unpaired samples.

Generation and characterization of C- and N-terminal Fra-2-specific antisera. Anti-Fra-2 sera were raised in rabbits against three synthetic peptides, which correspond to selected sequences present only in the Fra-2 polypeptide. The peptides used (and antiserum identification numbers) were as follows: rFra-$2_{68\text{-}96}$, VITSMSNPYPRSHPYSPLPGLRSVPQHM (2605); rFra-$2_{220\text{-}242}$, VVV KQEPPEEDSPSSSAGMDKTQ (2607); and rFra-$2_{286\text{-}296}$, PSVLEQESPAS (2612). For immunization, peptides were conjugated via branching on a lysine tree (3). The various antisera were evaluated by Western blot screening of rFra-2-transfected glioma C6 cell extracts. In this screening antibodies (Ab) 2605, 2607, and 2612 detected the same band (Fra-2 species) as that reacting with pan-Fos Ab F2P1 (36) (data not shown). Antisera were further characterized by peptide blocking analysis in supershift assays.

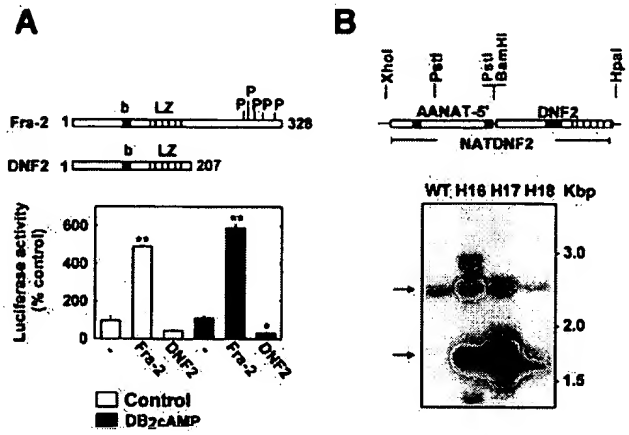

FIG. 1. Functional validation of the DNF2 design and transgene production. (A) The upper diagram depicts the rat Fra-2 protein (328 aa) (1). The basic DNA binding domain (b) and the leucine zipper (LZ) interaction regions are indicated. The carboxy-terminal region that undergoes the bulk of the regulatory phosphorylation events (aa 250 to 320) (28) is also marked (P). The DNF2 diagram shows the position of the truncation point relative to the functional Fra-2 domains. The bottom panel presents the luciferase activity results of transiently transfected NIH 3T3 cells. The reporter (1702/LUC [see Materials and Methods]) was cotransfected with the indicated mammalian expression vectors. Cultures were kept under control (open bars) or DB_2cAMP-stimulated (6 h) conditions before harvest. (B) Representative Southern analysis of rat genomic DNA. The diagram represents the NATDNF2 construct. In the bottom panel, genomic DNA samples (10 μg) from wild-type and NATDNF2 transgenic rats were digested with *Pst*I, resolved on a 1% agarose gel, and transferred onto a nylon membrane before probing with a ^{32}P-radiolabeled *Xho*I/*Bam*HI fragment of the transgene. This permits detection of an internal 1,703-bp fragment of the incorporated transgene (bottom arrow) and a 2.5-kb band representing the endogenous AANAT gene (upper arrow). Note that the latter band alone is detected in the WT sample. Other hybridizing bands represent random junction fragments derived from the transgene insertion sites. Size markers are a 1-kb DNA ladder (New England Biolabs, Beverly, Mass.).

EMSA and Western blot analysis. High-salt extraction for preparation of whole pineal cell extracts and electrophoretic mobility shift assay (EMSA) were performed as previously described (1). The ^{32}P-radiolabeled probe was an AP-1 consensus element (Promega). The following affinity-purified rabbit polyclonal antisera were used (Santa Cruz Biotechnologies, Santa Cruz, Calif.): SC253x (anti-pan-Fos), SC73x (anti-JunB), SC1694x (anti-c-Jun), and SC74x (anti-JunD). The Fra-2-specific antisera used (Ab 2605 and Ab 2607) are described above. For Western blot analysis, whole pineal cell extracts (~10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel, transferred onto nitrocellulose (Hybond-C; Amersham-Pharmacia), and exposed to a pan-Fos antibody as previously described (1).

Direct and reverse Northern blot analysis. Samples of total cellular RNA were resolved on formaldehyde-agarose gels and subjected to Northern blot analysis. Full-length cDNAs in plasmid vectors (2 μg) were denatured, immobilized onto a nylon membrane (Hybond N^+; Amersham-Pharmacia) using a slot blot apparatus (Schleicher & Schuell, Keene, N.H.), and hybridized with ^{32}P-radiolabeled cDNAs reverse transcribed from total pineal RNA samples (1 μg) using a preamplification kit (Life Technologies). Following a standard washing protocol, the blots were exposed to X-ray film (AX; Genetics Research Instruments, Essex, United Kingdom). The immobilized rat cDNAs (GenBank accession numbers and investigator source are indicated) were as follows: Fra-2 (U18913, R. Baler), c-Fos (X06769, T. Curran), AANAT (U38306, D. Klein), β1-adrenergic receptor (D00634, S. Coon), c-Jun (X17163, M. Iadarola), γ-phosphodiesterase (AF169390, S. Coon), α1A-adrenergic receptor (M60654, S. Coon), α1B-adrenergic receptor (X51585, S. Coon), preproenkephalin (Y07503, S. Sabol), type II iodothyronine deiodinase (DII) (U53505, D. Germain), S antigen (X15353, R. Baler), ICER (S66024, R. Baler), and RZRβ (NM_006914, R. Baler). Transgenic *fra-2* mRNA was detected by Northern blot analysis with a *fra-2*-specific

DNA probe derived from the last 500 bp of coding sequence. Endogenous *fra-2* mRNA was specifically detected with a 394-bp *Bsu*361/*Xba*I fragment which is missing in the transgene. DII mRNA was detected with a 1.4-kb *Eco*RI fragment derived from the rDII5-1 vector (Donald Germain, Dartmouth Medical School). Nectadrin mRNA was detected with a reverse transcription-PCR-generated, sequence-verified, 240-bp fragment of the rat cDNA (bases 102 to 342; GenBank accession no. NM_012752). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected with a 376-bp, sequence-verified, reverse transcription-PCR-generated fragment of the rat GAPDH cDNA (bases 556 to 932). 18S RNA was detected with a commercially available 1.2-kb probe (18S Decatemplate; Ambion, Austin, Tex.). Each Northern blot analysis sample consisted of pineal RNA that was pooled from two rats.

Array screen and analysis. Atlas 1.2 rat arrays (Clontech, Palo Alto, Calif.) were screened according to the manufacturer's protocol, using probes derived from 2 μg of total pineal RNA. A comprehensive list of the genes displayed on this array is available at http://www.clontech.com/atlas/genelists/RatTotal.txt. Each nylon array was probed three times using probes derived from different RNA samples, switching the probe (control versus transgenic) between consecutive screens. The data sets obtained were analyzed using the Atlas Image software (Clontech).

Animal care and experimental protocols. (i) Animal care. Rats (SD, 250 to 300 g) were entrained to a 12-h/12-h (12:12) light-dark schedule (lights on at 7 a.m.) for 2 weeks before experiments were carried out. Experiments were done in accordance with the Public Health Service policy on humane care and use of laboratory animals, *Guide for the Care and Use of Laboratory Animals* (33), and Animal Welfare Act regulations, following experimental protocols that were approved by the Animal Care and Use Committee and met the National Institutes of Health guidelines. No procedures performed during the course of this study caused more than momentary or slight pain or distress.

(ii) Urine collection experiment. For urine collection (Fig. 5), animals were maintained in individual, metabolic cages (Techniplast, Buguggiate, Italy) for an initial period of 2 days prior to the 24-h urine collection period. Urine volume was measured and 6-sulfatoxymelatonin was determined in aliquots (Stockgrand Limited, Surrey, United Kingdom).

(iii) Hypertonic challenge experiment. For the hypertonic saline experiment (Fig. 3), rats were sampled 3 h after either a hypertonic saline injection (1.5 M, 1.8 ml/100 g) or a similar injection of normal saline. Animals were killed by administration of a lethal injection of phenobarbital. Animals were then transcardially perfused with 50 ml of phosphate-buffered saline (pH 7.4) followed by 100 ml of 4% paraforamaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were carefully removed, postfixed in 4% paraformaldehyde (1 h, 4°C), and cryoprotected in 30% sucrose (4°C, 18 h). Brain cryosections (12 μm) containing the supraoptic nucleus (SON) were prepared; Fra-2 immunoreactivity was detected using a Vector ABC Elite kit (Vector Laboratories, Burlingame, Calif.). Ab 2612 (rFra-$2_{286-296}$) was the first antiserum (1:250, 4°C, 18 h). The second antiserum (peroxidase-conjugated, goat anti-rat; Jackson ImmunoResearch Laboratories, West Grove, Pa.) was detected using nickel-enhanced 3,3′-diaminobenzidine (Sigma, St Louis, Mo.), and brain sections were dehydrated and mounted without counterstaining. Sections were viewed with a Leica DM-LB microscope, photographed (Kodak 64-T film), scanned (Canon-Scan), and montaged (Adobe Photoshop, version 4.0).

RESULTS

Functional characterization of the truncated Fra-2 moiety. The negative dominance of the DNF2 moiety was evaluated by comparing its ability to transactivate the Fra-2 promoter relative to intact Fra-2 (Fig. 1A). The Fra-2 promoter represents a good candidate for a Fra-2 target in vivo because it contains two AP-1 sites that interact with a Fra-2-containing AP-1 complex (10, 50). Based on in vitro evidence (28), these sites have been proposed to mediate an autoregulatory Fra-2 transcriptional loop. A transient transfection assay revealed that truncation of the Fra-2 molecule at aa 207 (DNF2) prevented Fra-2-dependent stimulation of a Fra-2 promoter-driven reporter gene activity (Fig. 1A). This deficit was evident under both control and DB_2cAMP-stimulated conditions. Based on this, the DNF2 moiety was used to generate DNF2 transgenic rats.

Production and phenotypic analysis of pineal gland-specific DNF2 transgenic rats. Rat embryos were injected with a DNA restriction fragment carrying the AANAT promoter and the DNF2 sequence (Fig. 1B, diagram) as detailed in Materials and Methods. Transgene copy numbers in the resulting lines H4, H13, H16, H17, and H18 were estimated at 2, 5, 20, 45, and 4, respectively, per haploid genome (Fig. 1B, data for only WT and H16 to H18 are shown).

Transgenic founders and offspring did not exhibit overt phenotypes, and all offspring exhibited normal growth patterns. Direct weight comparisons were difficult to obtain because of the uneven distribution of transgenic mice within litters, but two littermate samples revealed no significant differences in weight (WT and transgenic 6-week-old males, 212 ± 9 g [n = 3] and 226 ± 8 g [n = 5], respectively; WT and transgenic 10-week-old females, 221 ± 2 g [n = 3] and 230 ± 4 g [n = 3]). Litters derived from mating hemizygote transgenic mice with WT mice displayed normal size and sex distribution. Histological analysis of transgenic tissues done as described in Materials and Methods revealed that the morphology of the H17 adult rat pineal gland was normal in that it was composed of a major population of homogeneous cell types exhibiting defined nuclei (presumed pinealocytes), together with a minor population of distinct cells and processes (presumed glia) and sympathetic nerve fibers. The retinas of H17 animals were also of WT appearance, with a normal ordering and density of cellular layers.

Tissue distribution of transgenic DNF2 expression and effects of its expression on the endogenous circadian Fra-2 response in the rat pineal gland. Tissue distribution of DNF2 was examined by Northern blot analysis with a *fra-2* probe; total RNA was extracted from several tissues from transgenic rats obtained at night. A robust DNF2 mRNA signal was detected in the night pineal gland; a weaker signal was detected in the retina (Fig. 2A). DNF2 mRNA was undetectable in the other tissues tested. This confirms that the AANAT promoter region used confers a high degree of tissue-specific gene expression (4).

The steady-state levels of pineal *fra-2* and DNF2 mRNA were determined in WT and transgenic animals that were sacrificed at various times during a 12:12 light-dark cycle. A robust 24-h rhythm in DNF2 mRNA levels was detected in NATDNF2 rats, with high levels at night. In contrast, the levels of endogenous *fra-2* mRNA were significantly lower at night than for control rats (Fig. 2B).

Western blot analysis using a pan-Fos antiserum revealed that Fra-2 protein increased steadily throughout the night in pineal glands from WT animals (Fig. 2C, lanes 1 to 4), confirming previous observations (1). In contrast, the levels of 67- and 35-kDa pan-Fos-immunoreactive species, corresponding to c-Fos and FosB, did not change significantly. These results are in agreement with previous observations (1). In contrast, the Fra-2 response in NATDNF2 animals was suppressed and inversely proportional to the level of DNF2 protein (22.7-kDa predicted molecular mass), which was detected as a ~20-kDa pan-Fos-immunoreactive band (Fig. 2C, lanes 5 to 8).

NATDNF2 transgenesis fails to perturb a nonpineal Fra-2 response. To validate the pineal gland (and retina)-specific nature of the NATDNF2 model, expression of the *fra-2* gene was examined in another tissue that expresses this gene, the

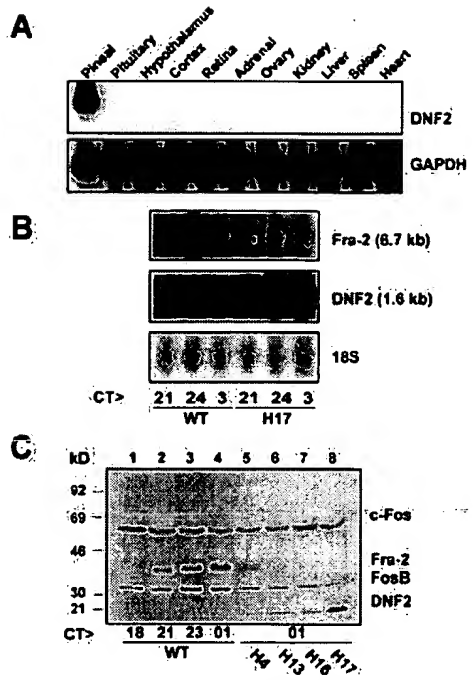

FIG. 2. Tissue survey of DNF2 expression and effects of pineal expression on the Fra-2 response in the night pineal gland. (A) Northern blot analysis performed with total RNA (~5 μg) extracted from the indicated tissues that were harvested in the middle of the night from transgenic animals designated H17 and carrying a high copy number (estimated at 45 per haploid genome) of the NATDNF2 transgene. The membrane was probed with Fra-2 and GAPDH radiolabeled probes. Blots were exposed to X-ray film for 24 h. No DNF2 signal was observed in WT animals (not shown). (B) Northern blot analysis of total pineal RNA (~7 μg) was used to investigate the nocturnal Fra-2 response in WT animals and in a high-copy-number transgenic line (H17). Levels of *fra-2* and DNF2 mRNAs were assessed at three different time points during the night along with the levels of the 18S rRNA. Downregulation of nocturnal *fra-2* mRNA expression was also observed in an independent experiment using samples derived from H16 transgenic rats. CT, clock time (hours). (C) Western blot analysis of pineal proteins prepared from WT mice or animals carrying a range of DNF2 transgene copy numbers (2 [H4], 5 [H13], 20 [H16], and 45 [H17] copies per haploid genome). Animals were sacrificed at the indicated clock times, and SDS-PAGE was run with total pineal protein samples. After electrophoretic transfer, the membrane was exposed to a pan-Fos rabbit antibody directed against the invariant M peptide between aa 129 to 153 in the mouse c-Fos sequence (SC253x). For both panels, the light-dark cycle was 12:12 with lights off at 7 p.m. Selective downregulation of Fra-2 protein was observed in two independent experiments using samples derived from additional H17 transgenic rats.

SON. The SON displays a low and constitutive level of *fra-2* expression (40) as well as a robust Fra/AP-1 response to neuronal hyperactivity and hyperosmolality (44, 47, 49). Expression of Fra-2 was detected by immunocytochemistry using an antibody (Ab 2612) directed against a peptide corresponding to Fra-$2_{286-296}$, which is absent in the DNF2 protein. It was found that the levels of endogenous Fra-2 induction in the WT and transgenic SONs were similar (compare Fig. 3B and D). This result is in sharp contrast to the marked suppression of the pineal Fra-2 response in NATDNF2 animals (Fig. 2B and C) and provides further evidence of the selective power of this strategy for modulating the expression of a target gene in a highly tissue-specific manner.

Binding characteristics of DNF2 protein. In vivo-expressed DNF2 protein was examined by EMSA to determine whether it displays the predicted DNA binding pattern and obeys the same heterodimerization rules as intact Fra-2. Fra-2 and truncated Fra-2 proteins in the AP-1 complex were differentially detected using supershifting antibodies. Ab 2605 detects the amino terminus of Fra-2, which is also present in the DNF2 protein; Ab 2607 detects the carboxy terminus of Fra-2, which is absent in the DNF2 protein (Fig. 4A).

Most of the AP-1–nucleoprotein complex in control and NATDNF2 pineal extracts obtained at night was supershifted by Ab 2605 (Fig. 4B, left), consistent with the fact that Ab 2605 cannot distinguish between full-length and truncated Fra-2. In contrast, Ab 2607 supershifted the complexes derived from WT but not those from NATDNF2 pineal glands (compare C4 and C17 with H4 and H17, respectively), consistent with its recognition of an epitope present in the WT protein but not in the truncated form.

It should be noted that the low-copy-number line H4 still contains residual endogenous Fra-2, as revealed by the small amount of Ab 2607-immunopositive material (see also Fig. 2A, lane 5). Specificity of the antibodies was confirmed by the ability of the immunizing peptides to selectively block the supershift caused by their cognate antibodies (Fig. 4B, center) and by their inability to supershift all AP-1 complexes, a small percentage of which does not appear to involve Fra-2.

The DNF2 protein appears to heterodimerize with Jun proteins, based on evidence that the same supershift profiles were obtained with each of three anti-Jun antibodies using either WT (C17) or NATDNF2 (H17) pineal extracts (Fig. 4B, right).

Identification of putative Fra-2 target genes. The finding that transgenic NATDNF2 rats do not exhibit a detectable pineal Fra-2 response suggests that DNF2 protein may act in part by inhibiting *fra-2* transcription. This leads to the possibility that the DNF2 moiety might also inhibit transcription of other genes that are regulated through similar mechanisms. This was tested in several ways.

(i) AANAT and melatonin production. We first focused on expression of AANAT and melatonin production, which have been suggested to be controlled by Fra-2 (1, 13). Northern blot analysis (Fig. 5A) indicated that AANAT mRNA levels in control and DNF2 animals were essentially identical. Examination of Fos proteins using the pan-Fos antibody failed to detect any changes in the expression of proteins carrying the pan-Fos epitope, making it unlikely that enhanced expression of a related member of the Fos family compensated for the lack of intact Fra-2 in this system (Fig. 2C).

Expression of other genes involved in melatonin production and its major metabolic derivative was examined by monitoring urinary 6-sulfatoxymelatonin. The 24-h patterns in this parameter were found to be essentially identical in the WT and NATDNF2 animals, consistent with the interpretation that Fra-2 does not play an essential role in the pineal gland in melatonin production.

(ii) Screening of selected pineal genes. A second approach was to examine the nocturnal increase in the expression of selected genes that are known to be under circadian/noradrenergic control in the rat pineal gland. Plasmids carrying the

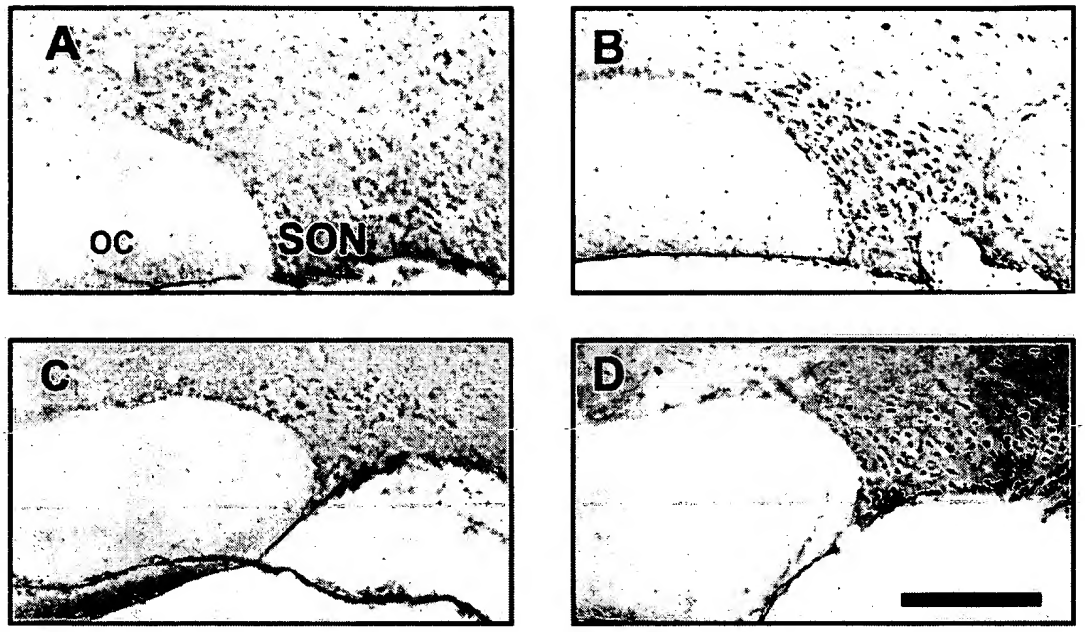

FIG. 3. Hypertonic saline induction of Fra-2 in the SON of WT and NATDNF2 rats. WT (A and B) and H17 NATDNF2 (C and D) rats (250 to 300 g) were sampled 3 h after either a hypertonic saline (1.5 M) injection (B and D) or a similar injection of normal saline (A and C). Endogenous Fra-2 in the SON was detected by immunocytochemistry using carboxy-specific rFra-2 Ab 2612. Sections were viewed with a Leica DM-LB microscope, photographed (Kodak 64-T film), scanned (Canon-Scan), and montaged (Adobe Photoshop, version 4.0). OC, optic chiasma. Scale bar = 250 μm.

coding sequences for 13 different genes (Fig. 6) were immobilized on duplicate membranes. The membranes were subsequently probed with cDNA derived from WT (C17) or transgenic (H17) pineal RNA that had been harvested in the middle of the night. Several genes represented on this screen displayed an apparent upregulation in the NATDNF2 line; however, only the DII gene showed a reproducible and significant difference in NATDNF2 animals (Fig. 6A, sample 11). The reverse Northern data were confirmed and expanded through a Northern blot analysis showing that DNF2 expression causes a >2-fold increase in the amplitude of the nocturnal increase in DII mRNA (Fig. 6B and C). Interestingly, this effect of DNF2 is in the opposite direction relative to the suppressive effect on *fra-2* mRNA levels, supporting the notion that Fra-2 can exert opposite effects in transcriptional control.

(iii) Screening of an array. A third approach for the identification of Fra-2 target genes involved the probing of a cDNA array with WT (C17) and transgenic (H17) cRNA pools. A comprehensive array screen is not possible at this time. However, it was of interest to examine the general utility of this approach and to determine if Fra-2-regulated genes were abundant or rare. To do so, we used cRNA prepared from pineal glands harvested at midnight (Fig. 7A). A small fraction of the 1,176 genes represented on the array showed a difference between WT- and NATDNF2-derived probes. Out of this initial pool of candidates expression of nectadrin, also referred to as CD24 (42), exhibited the lowest transgenic/control ratio. This was subsequently confirmed by Northern blot analysis (Fig. 7B and C). Accordingly, it appears that nectadrin levels are markedly reduced in the pineal gland of NATDNF2 transgenic animals.

DISCUSSION

Experimental strategy. Two genetic approaches for suppressing Fra-2 expression in the rat pineal gland were considered at the onset of this study, one involving antisense cDNA and the other based on the generation of a DN protein. The antisense approach has been successful in knocking out expression of target genes (20). However, this strategy was rejected because it typically requires extensive empirical optimization of the target region along the mRNA molecule; this becomes an insurmountable obstacle when the target gene belongs to a family of closely related members with partial homologies throughout their coding sequences. Furthermore, antisense oligonucleotides can lead to unexplained effects when introduced into the cell.

A DN strategy was selected because it has also been widely used, with notable success in the analysis of cAMP response element (CRE) and AP-1 DNA binding proteins (14, 27), and DN molecules that display a high level of specificity can be easily engineered. DN transcription factors are often designed so that they retain the ability to heterodimerize with specific partners but lack a DNA binding domain, which precludes them from interacting with their cognate regulatory sequence along the promoter (23). As a result, this class of DN molecules affects transcription output via a squelching effect. A second class of DN molecules lacks the *trans*-acting domain. These proteins retain the ability to heterodimerize and bind to DNA but lack transcriptional signaling (16).

This led to the design of the DNF2 molecule, which had dramatically reduced *trans*-acting potential compared with the full-length protein in an AP-1-driven system (Fig. 1A). This

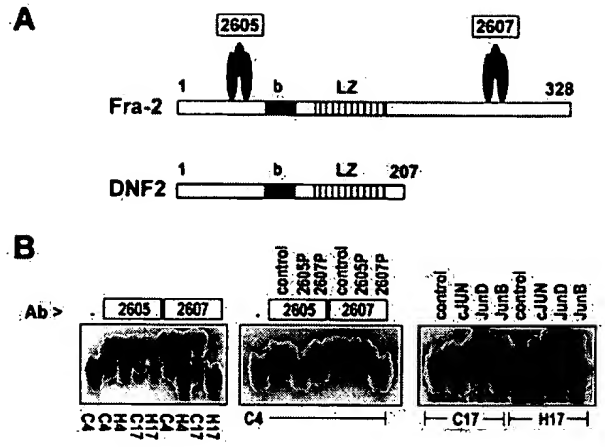

FIG. 4. The DNF2 molecule maintains the proper DNA binding and heterodimerization patterns. (A) Two rabbit polyclonal antisera were raised against the rat Fra-2 molecule. Ab 2605 recognizes rFra-2_{68-96}, while Ab 2607 is directed against Fra-$2_{221-244}$. Thus, use of Ab 2607 serves to distinguish between full-length and truncated Fra-2. (B) EMSA supershift analysis of nocturnal pineal extracts prepared from transgenic (H4 and H17) and control (C4 and C17) littermates. Sera used were anti-Fra-2 Ab 2605 and 2607 (left and center) and anti-c-Jun, -JunD, and -JunB (right). Specific inhibition of Fra-2 supershifts was performed with the indicated peptide at a final concentration of 50 μM (center). Results were confirmed in an independent experiment using pineal glands derived from additional H17 rats.

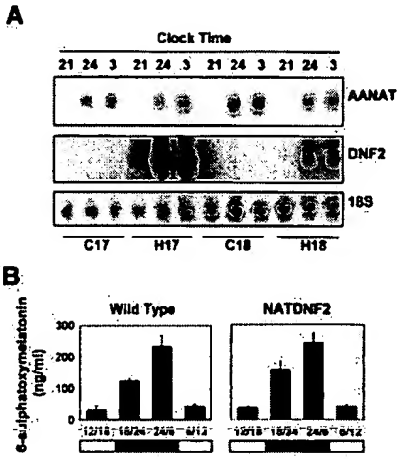

FIG. 5. Expression of DNF2 protein has no effect on pineal AANAT expression or melatonin production. (A) Northern blot analysis of total pineal RNA (~7 μg) extracted from WT and NATDNF2 animals that were sacrificed at the indicated clock times throughout a 24-h cycle. Blots were exposed to rat AANAT, DNF2, and 18S probes. The absence of transgene-associated changes in nocturnal AANAT mRNA level was confirmed in an independent experiment using samples derived from additional H17 transgenic rats. There were six rats in each group. (B) WT and H17 NATDNF2 rats were maintained in individual metabolic cages on a 12:12 light-dark cycle (lights off at clock time 19). Urine was collected every 6 h, starting at clock time 12, and urinary 6-sulfatoxymelatonin (expressed as total nanograms produced/period) was assessed for each group.

confirms the importance of the carboxy-terminal phosphorylation sites in supporting Fra-2 activity (28). It is also relevant to point out that NIH 3T3 cells appear to be deficient in their capacity to activate the *fra-2* (this study) or c-*fos* gene in response to DB_2cAMP activation (43), presumably due to the lack of Ser-133 phosphorylation of the CRE binding factor CREB (43). This phenomenon allowed us to better dissect the Fra-2-dependent component of *fra-2* gene induction.

Selective expression in the pineal gland was achieved by putting the DNF2 behind the AANAT promoter, an approach which has been found to target expression of a reporter transgene to the pineal gland and, to a lesser degree, the retina (4). DNF2 mRNA was abundant in the pineal gland of several lines of NATDNF2 rats but not in other tissues except the retina, in which DNF2 expression was detectable at low levels. Pineal DNF2 mRNA levels changed following a 24-h rhythm, with high levels at night, as is typically seen with AANAT expression. Accordingly, this effort appears to have successfully achieved the goal of engineering an animal expressing the DNF2 gene in the pineal gland under the control of the AANAT promoter. It was also found that expression of the DNF2 gene resulted in the production of DNF2 protein (Fig. 2C). Importantly, the overall patterns of Jun heterodimerization and high-affinity AP-1 binding supported by DNF2 were comparable to those displayed by the full-length Fra-2 (Fig. 4B). This indicates that the DNF2 molecule assumed the correct conformation required for binding, even though it was a truncated form of the parent molecule. Interestingly, the residual and relatively constant level of AP-1 DNA binding activity not affected by the Fra-2 antibodies suggests that neither low nor high levels of DNF2 overexpression can efficiently compete for Jun heterodimeric partners, yet some level of competitive inhibition cannot be ruled out.

Evidence of positive autoregulation of Fra-2 expression. As indicated above, it is known that expression of Fra-2 in the rat pineal gland increases at night, resulting in ~200-fold-higher levels of *fra-2* mRNA and protein. In the present study we found that expression of Fra-2 was suppressed in the DNF2 pineal gland, consistent with the existence of positive autoregulation (28). Fra-2 expression was low in lines with high (H17) or low (H4 and H13) copy numbers of the transgene. The observation of similar effects, with either high or low transgene copy numbers, argues against the likelihood of nonspecific effects due to overexpression of the DNF2 transgene and supports the interpretation that effects of the transgene involve Fra-2 specifically.

The existence of a Fra-2 positive autoregulatory loop was first proposed based on the ability of phosphorylated Fra-2 to induce *fra-2* gene expression in vitro (28). Our observations that cotransfected DNF2 protein failed to stimulate *fra-2* promoter-driven reporter gene activity whereas intact Fra-2 has this effect are fully consistent with a model involving autostimulation (Fig. 1A). Accordingly, the present study has provided strong support for the view that the *fra-2* gene is under the control of Fra-2 protein in vivo. The evidence that expression of the *fra-2* gene is subject to positive feed-forward autoregulation at the transcriptional level is in agreement with the hypothesis that elevated levels of Fra-2 protein are required to sustain high levels of *fra-2* transcription in vivo (26). The initial ripples in the Fra-2 wave might be required to sustain the transcriptional response at a high level.

The basis for the strong correlation between DNF2 expres-

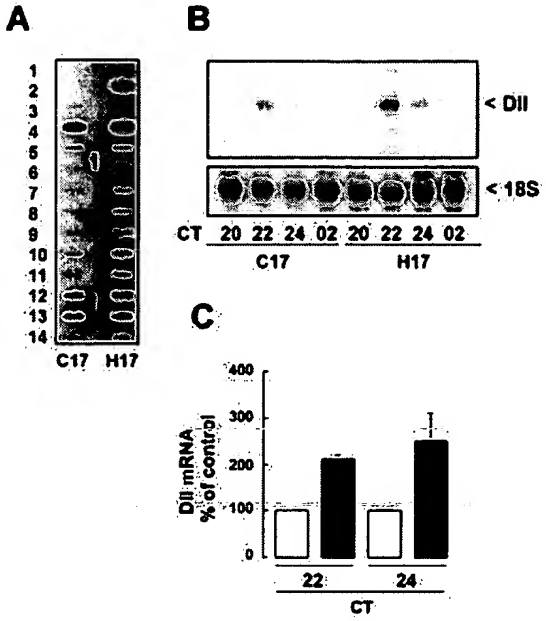

FIG. 6. DII is a candidate target gene for Fra-2 action. (A) Reverse Northern analysis of radiolabeled pineal (midnight) cRNA from WT and NATDNF2 rats. The immobilized plasmid vectors were as follows: 1, pUC18; 2, Fra-2; 3, c-Fos; 4, AANAT; 5, β1-adrenergic receptor; 6, c-Jun; 7, γ-phosphodiesterase; 8, α1A-adrenergic receptor; 9, α1B-adrenergic receptor; 10, preproenkephalin; 11, DII; 12, S antigen; 13, ICER; 14, RZRβ. All expression vectors contain rat genes. (B) Northern blot analysis of total pineal RNA (~7 μg) extracted from WT and NATDNF2 animals that were sacrificed at the indicated clock times (CT) throughout the dark phase of a 24-h cycle. Blots were exposed to rat DII and 18S ribosomal probes. (C) Quantitation of the relative abundance of the DII/18S steady-state transcript levels at 22 (n = 3 pairs of rats) and 24 (n = 4 pairs of rats) h. Open bars, WT; closed bars, NATDNF2.

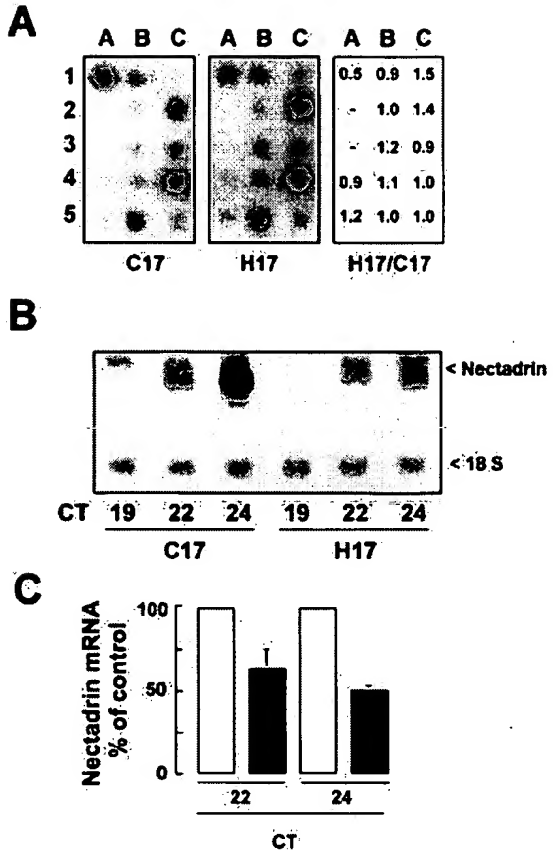

FIG. 7. cDNA array-based identification of nectadrin as a candidate Fra-2 target gene. (A) Representative details of duplicate rat expression arrays (Clontech's Atlas rat 1.2) probed with cDNAs derived from either transgenic (H17) or control (C17) littermate pineal glands sampled at 24 h. The nectadrin gene is located at position 1A. Following hybridization and washing, arrays were exposed to a storage phosphor screen (Kodak-K) for 3 days and visualized using a model FX molecular imager (Bio-Rad). Images were downloaded as TIFFs and montaged using Adobe Photoshop, version 4.0. The level of nectadrin mRNA is significantly reduced at midnight in the pineal gland of NATDNF2 rats. (B) Nectadrin expression displays a robust day/night rhythm in the rat pineal gland, as determined by Northern blot analysis of total pineal RNA (~7 μg) extracted from WT and NATDNF2 animals that were sacrificed at the indicated clock times (CT) throughout the dark phase of a 24-h cycle. Blots were exposed to rat nectadrin and 18S probes. (C) Quantitation of the relative abundance of the nectadrin/18S steady-state transcript levels at 22 and 24 h (n = 3 pairs of rats in each group). Open bars, WT; closed bars, NATDNF2. Statistical analysis for both DII and nectadrin levels of expression was performed by an unpaired t test, using corrected percentages as input values. The DII level is different from control at both 22 and 24 h, while the nectadrin is different from control at only 24 h, at a significance level of $P < 0.05$.

sion and Fra-2 suppression is not entirely clear. The increase in pineal Fra-2 at night reflects the action of cAMP and may involve CRE sites in the *fra-2* promoter. Fra-2 positive autoregulation could occur if Fra-2 acted through the previously identified AP-1 sites in the *fra-2* promoter (9) to enhance cAMP-initiated expression of the *fra-2* gene. In this model, *fra-2* transcription would be reduced in the absence of Fra-2 protein, which in turn could contribute to low levels of Fra-2 protein. Other hypothetical mechanisms through which the DNF2 moiety could suppress Fra-2 protein levels include those that enhance degradation, such as competitive suppression of Fra-2 heterodimerization or specific posttranslational modifications. Additionally, it remains plausible that the functions of other Fos family members may be regulated posttranscriptionally.

Although the molecular mechanism thorugh which the DNF2 moiety suppresses *fra-2* expression is not clear, it is apparent that the NATDNF2 rat model provides a unique opportunity to identify putative Fra-2 target genes, because the nocturnal increase in Fra-2 does not occur in their pineal glands. The finding that *fra-2* gene expression in the DNF2 pineal gland is suppressed provides further reason to expect that the DNF2-based strategy would identify other putative Fra-2 target genes. Accordingly, we initiated an effort to identify such targets by screening for genes whose nocturnal expression patterns become disrupted in the pineal gland of NATDNF2 animals.

Identification of putative Fra-2 gene targets. Two approaches were used to identify putative Fra-2 gene targets. The first used direct or reverse Northern blot analysis to examine a small group of likely candidate targets that are expressed in the pineal gland on a rhythmic basis; the second used array technology to examine a large group of broadly expressed genes.

Most notable among the pineal targets was the AANAT gene itself, which had been proposed to be a Fra-2 target gene on the basis of considerable circumstantial evidence (1, 13). However, our analysis failed to provide any evidence that Fra-2 is an essential component of regulation of the AANAT gene or of any gene involved in melatonin production. This result highlights the important role that direct genetic approaches such as the DN strategy can play in analyzing gene expression and why they are essential in studies of this nature.

Another member of the group of selected candidates was DII, which is thought to activate circulating iodothyronine at discrete sites of action (41). The levels of DII mRNA and enzyme activity increase at night in the rat pineal gland (18, 29). Transcription of the DII gene is under circadian/adrenergic→cAMP regulation in this tissue (18), as is true for *fra-2* and the AANAT gene. The finding in this study that the nocturnal increase in DII mRNA was >2-fold greater in NATDNF2 animals suggests that DII is subject to negative regulation by a Fra-2-containing AP-1 binding complex and that Fra-2 may in some way limit expression of DII. The likelihood that an AP-1 site is involved is supported by the presence of several putative AP-1 sites in the human DII gene promoter (5). Further investigation will be necessary to determine if similar sites are present and functional in the rat DII gene and whether they are involved in the Fra-2-mediated downregulation of this gene in the rat pineal gland.

The second group of genes examined numbered 1,176. Differential screening of these genes allowed for direct comparison of the expression patterns in NATDNF2 and WT animals. This approach identified another gene that appears to be positively regulated by Fra-2, nectadrin, also known as CD24 (8). Nectadrin is a glycosylphosphatidylinositol anchored adhesion molecule that is expressed in hematopoietic and neural cells; pineal expression of CD24 has not been reported. Furthermore, this gene displays a rhythmic pattern of expression in the rat pineal gland; its peak level at midnight is significantly reduced in NATDNF2 animals (Fig. 7B and C). To date, CD24 has been functionally implicated in control of cell-cell and cell-substrate binding as well as in Src kinase-associated intracellular Ca^{2+} signaling (39). Although nectadrin might play a role in signal transduction in the pineal gland, it is premature to speculate on this possibility. The identification of nectadrin as a rhythmically regulated gene in the pineal gland by the methods described here highlights the power of this approach for opening novel avenues of research.

Significance and future directions. The rat pineal gland is an excellent model to investigate the transcriptional role of Fra-2. The circadian pattern of Fra-2 expression in the pineal gland is of special interest because of the ~200-fold magnitude of the nocturnal increase combined with the evidence that expression of other members of the Fos family does not change significantly during a 24-h period. The increase in pineal Fra-2 has been shown to rely heavily on the second messenger cascade triggered by norepinephrine signaling (Fig. 8). This cascade leads to the phosphorylation of CREB (21) and the activation of the MAPK pathway (17). The former is responsible for the nocturnal induction of *fra-2* and many other pineal genes, while the latter is known to effect massive Fra-2 phosphorylation (11, 28). It is conceivable that Fra-2 could function as a transcriptional sensor to integrate the activation level of these

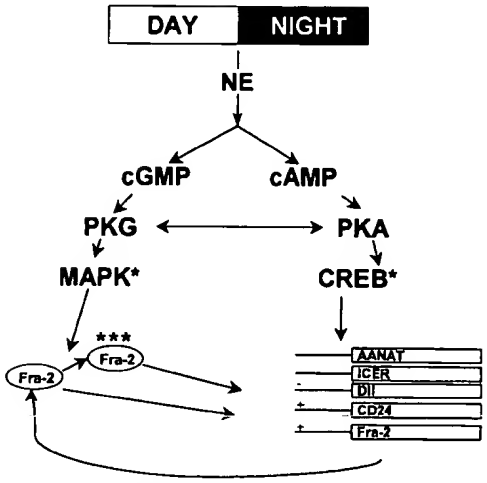

FIG. 8. Hypothetical model of the central location of the Fra-2 transcription factor within the noradrenergic signaling cascade in the rat pineal gland. Fra-2-containing AP-1 complexes can select different adrenergically regulated genes to either drive their expression further up (+) or limit the strength of their response (−). Many genes, however, are not affected by the Fra-2 knockdown, even though their promoters contain putative AP-1 sequences. Likely connections between the protein kinase A (PKA) and PKG pathways (17) are indicated by a dashed double arrow and are poised to affect the levels and phosphorylation status of a Fra-2-containing AP-1 factor in complex ways. NE, norepinephrine.

two (possibly more) concomitant signal transduction pathways. Thus, based on the accumulated data and the results of the present in vivo study, it seems reasonable to hypothesize that the overall levels of Fra-2, coupled to the ratio between hyper- and hypophosphorylated Fra-2, might constitute a transcriptional rheostat ideally placed to fine-tune the sensitivity of different AP-1-responsive genes to the adrenergic stimulus in both directions. The vast majority of genes (e.g., AANAT and ICER) will remain unaffected, and others (e.g., Fra-2 and nectadrin) require Fra-2 action to maintain a high level of expression, while a third group (DII-like) might be actively repressed at night by a Fra-2-containing AP-1 complex.

It will be of great interest to take advantage of this rat pineal transgenic system to attempt and dissect the molecular basis for the bimodal capacity of a Fra-2-containing AP-1 complex to modulate transcriptional events. In this context, analysis of the differential recruitment of specific chromatin-remodeling complexes on different Fra-2-decorated promoters delineates a fertile and intriguing possibility (48).

This study represents a proof of principle for the transgenic delivery of a DNF2 transcription factor using a highly tissue-specific promoter in the rat system. Analysis of more densely populated gene arrays from a variety of sources will undoubtedly reveal a much higher complexity in the network of downstream genes, both known and unknown, potentially regulated by a Fra-2-containing complex, either positively or negatively.

Finally, the results of this study indicate that the use of a DNF2 moiety, in combination with novel tissue-specific promoters, may prove to be of value in the analysis of Fra-2 biology throughout development (6) and in selected adult structures where robust responses in its expression occur, in-

cluding the hippocampus (19, 35), suprachiasmatic nuclei (40), and adrenal medulla (32).

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